1118

Photooxidation of Crystalline *Clostridium botulinum* Type A Toxin in the Presence of Methylene Blue

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Introduction

The action of visible light in the presence of dyes on certain viruses and toxins has been studied previously (1–5). These investigations were carried out on impure material, and the degree of photooxidation was not followed quantitatively. Although in all cases a rapid destruction of biological activities was observed, the resulting products differed in respect to their antigenic properties. As an extension of our previous work on the action of sensitized visible light on crystalline enzymes (6–8), we have studied the manner in which various degrees of photooxidation affect the biological and immunological characteristics of crystalline botulinum type A toxin (9, 10).

Experimental

The crystalline botulinum type A toxin used for the irradiation studies was prepared by differential ultracentrifugation and salting-out with ammonium sulfate.[3] This preparation contained 226×10^6 mouse LD_{50}/mg. protein nitrogen, essentially the same potency as reported previously in the literature (9, 10).

The degree of photooxidation was followed by measuring the oxygen uptake at 37°C. by the manometric Warburg procedure (11). In all cases, the center well of the Warburg vessel contained concentrated KOH in order to adsorb the CO_2 simultaneously evolved during the photooxidation of the toxin.

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[1] This work was performed for the Chemical Corps through Interagency Agreement between Fort Detrick, Maryland and U. S. Department of Agriculture.

[2] A laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[3] Spero, L., and Schantz, E. J., unpublished results.

Effect of Photooxidation on the Toxicity of Botulinum Type A Toxin

To determine the effect of photooxidation on the toxicity of botulinum type A toxin, the following procedure was used. Three milliliters of solution, containing 5.25 mg. of the toxin, buffered with 0.4 *M* phosphate at pH 7.0, was placed in the main chamber of the Warburg vessel, while 0.5 ml. of a 0.02% solution of methylene blue was placed in the side arm. After temperature equilibrium, the two were mixed and irradiation with visible light was started. After the desired oxygen uptake was reached, the residual toxicity was measured by injecting 3.2-fold dilutions of the toxin (in 0.2% gelatin diluent containing 1% phosphate buffered at pH 6.8) intraperitoneally into mice, six mice being used at each concentration. Titers were determined after 4 days by a graphical probit method plotting logarithm of dose against per cent killed (12). The results obtained and presented in Table I show the rapid detoxification of the toxin with progressive photooxidation:

TABLE I

Influence of Photooxidation of Botulinum Type A Toxin on Toxicity

O_2 uptake/5.25 mg. of toxin *cu. mm.*	Mole of O_2/mole of toxin[a]	Irradiation time *min.*	Toxicity, LD_{50}/ml.	Decrease in toxicity %
0	0	0	55.5×10^6	0.00
3	23	5	6.5×10^6	88.3
5	39	10	0.8×10^6	98.6
8	62	13	0.33×10^6	99.4
12	93	19	0.033×10^6	99.94
30	232	42	5.5×10^2	99.999

[a] Calculated on the basis of 900,000 as the molecular weight of the toxin.

Irradiation carried out under identical conditions as described for the experiments listed in Table I, but in the absence of the dye, showed no decrease in toxicity. Similarly, no decrease in toxicity was observed if the dye was added to the toxin but the mixture was kept in the dark prior to the toxicity measurements.

Effect of Photooxidation of Botulinum Type A Toxin on the Reaction with Its Homologous Antiserum

Experimental conditions for the irradiation of the toxin were identical to those described in the preceding section. After the desired degree of

toxin photooxidation, its combining power with the homologous antiserum was measured by the Heidelberger and Kendall precipitin reaction (13).

To 1-ml. aliquots of botulinum type A horse antiserum (Lederle antiserum; 500 units/ml., used in 1:4 dilution), the toxin or irradiated toxin was added in increasing concentration and the mixture was made up to a volume of 2 ml. After 2 hr. incubation at 37°C., the precipitate which formed was centrifuged, the sediment was washed three times with 1 ml. of saline solution, and the total N in the precipitate was determined. The equivalence zone was determined by testing the supernatant for excess of antigen and antibody by the ring test.

When the reaction was carried out at 4°C. instead of at 37°C., an increase in precipitate formation between antigen and antibody was observed. This confirms the original observation of Lamanna and Doak (14), which was ascribed to the precipitation of normal serum constituent by the toxin in addition to the antibody at low temperature.

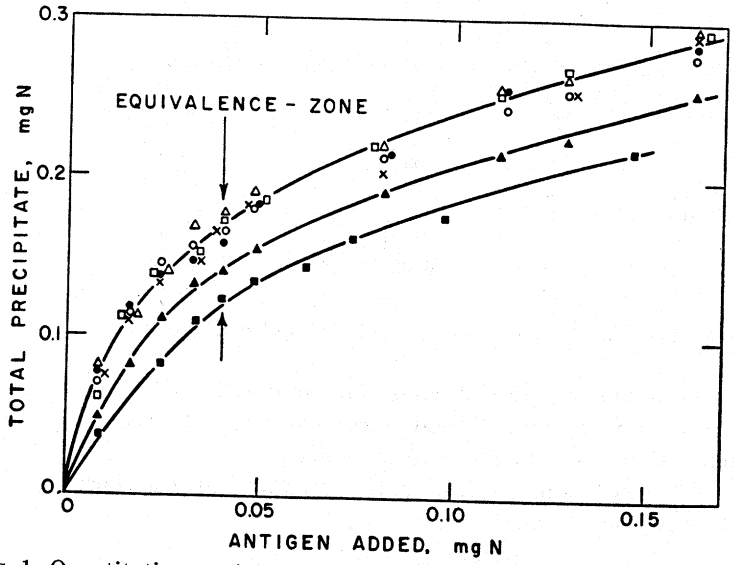

FIG. 1. Quantitative precipitin reaction with botulinum type A antiserum and native or photooxidized toxin.

O—O native toxin; ●—● 3 cu. mm. O_2 uptake;
×—× 5 cu. mm. O_2 uptake; △—△ 8 cu. mm. O_2 uptake;
□—□ 11 cu. mm. O_2 uptake; ▲—▲ 24 cu. mm. O_2 uptake;
■—■ 34 cu. mm. O_2 uptake
(All O_2 uptake figures refer to 5.25 mg. toxin.)

TABLE II

Quantitative Precipitin Reaction of Native and Photooxidized Toxin with Antitoxin[a]

Toxin added	Native toxin			Photooxidized toxin (24 cu. mm. O_2)			Toxin added	Photooxidized toxin (34 cu. mm. O_2)		
	Total pptd.	Supernatant		Total pptd.	Supernatant			Total pptd.	Supernatant	
		Antitoxin	Toxin		Antitoxin	Toxin			Antitoxin	Toxin
μg. N	*μg. N*			*μg. N*			*μg. N*	*μg. N*		
8	70	+	0	48	+	0	8	30	+	0
16	115	+	0	80	+	0	24	83	+	0
24	145	+	0	108	+	0	32	105	+	0
32	151	+	0	130	+	0	40	110	0	0
40	165	0	0	142	0	0	48	134	0	0
48	180	0	+	154	0	0	61	142	0	+
81	214	0	+	192	0	+	73	163	0	+
113	245	0	+	212	0	+	97	175	0	+
130	251	0	+	225	0	+	146	220	0	+
162	282	0	+	258	0	+				

[a] The precipitin reaction of photooxidized toxin (3–11 cu. mm. O_2 uptake) with the antitoxin gave essentially identical results as those obtained with the native toxin.

The inhibition zone usually observed in the region of antigen excess was absent in our experiment, as was the case with the previous findings of Lamanna and Doak (14). The results obtained and presented in Fig. 1 and Table II show that the combining power of botulinum type A toxin with its homologous antiserum was not affected up to 11 cu. mm. of oxygen uptake/5.25 mg. toxin, and that only moderate reduction was observed at 24 and 34 cu. mm. of oxygen uptake. Photooxidation of the toxin beyond this point, however, resulted in a precipitate formation (denaturation), which made the application of the above procedure mpossible.

Irradiation of the toxin under identical conditions, but in the absence of the dye, resulted essentially in the same precipitin curve as the original toxin.

For preliminary antigenicity studies, the detoxified material was adsorbed on aluminum phosphate, and 1-ml. aliquots of various dilutions were injected subcutaneously using five mice per dilution. All challenges were made against 15 LD_{50} dose 2 weeks after the last immunizing dose.

In the first series of experiments, 5.25 mg. of toxin was photooxidized to the extent of 26 cu. mm. of O_2. With this detoxified sample 50% protection was achieved if three weekly doses containing 0.6 μg. of toxin N each were administered.

In a second series of experiments, 5.25 mg. of toxin was photooxidized to the extent of 9 cu. mm. of O_2. Since the residual toxicity of this preparation amounted to about 0.3% of the original, it was treated with 0.2% of formaldehyde for 24 hr., and administered as above. Under these conditions 50% protection was obtained by a single dose of 1.6 μg. of toxin N, while if the immunization was carried out in three weekly doses, the amount required for each injection was 0.4 μg. of toxin N. Control experiments have demonstrated that the minute amount of toxicity present in the above preparations did not produce any protection against the challenge dose.

Discussion

Previous studies of the inactivation of various enzymes by sensitized visible light (6–8) have proved to be useful in correlating catalytic activity with chemical structure, and have emphasized that the imidazole side chain of the enzyme molecule is important to activity.

It has been found in this study that groups responsible for the toxic action of crystalline botulinum type A toxin were markedly affected by irradiation, and that a very low level of oxygen uptake by this toxin molecule was sufficient to eliminate practically all its toxicity (12 cu. mm. of oxygen uptake/5.25 mg. toxin resulted in 99.94% reduction of the toxicity).

Detoxification of botulinum type A toxin by photooxidation did not result in a reduction of its combining power with the toxin antibody *in vitro*, and only at 24 cu. mm. of oxygen uptake/5.25 mg. toxin was a moderate reduction in the precipitin curve observed.

In view of the fact that at present no analytical data are at hand to correlate the rapid decrease in toxicity due to photooxidation with changes produced in the amino acid composition of this toxin, no conclusion is possible as to the chemical nature of the toxic groups. One might point out, however, that in previous work on enzymes (6–8), without exception, it was the imidazole side chain of the enzyme molecule which was preferentially photooxidzed. The possibility that this side chain might be related to the toxicity of botulinum toxin, although suggestive, is highly speculative at this time.

Preliminary experiments on the antigenic nature of the photochemically detoxified toxin appear to be promising, but further experiments are required to prove the usefulness of this method for toxoid production.

Summary

Photooxidation of crystalline botulinum type A toxin in the presence of traces of methylene blue results in a very rapid detoxification of the toxin.

The combining power of the photochemically produced toxoid with the toxin antibody *in vitro* was not reduced as compared to the original toxin. Only a more extensive photooxidation of the toxin resulted in a moderate reduction of this property.

Preliminary tests indicate that the protein detoxified by means of photooxidation is antigenic.

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